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Immunobilization of Barley Oxalate Oxidase onto Alkylamine Glass for Determining Urinary Oxalate

To the Editor:
Enzymatic colorimetric determination of urinary oxalate by oxalate oxidase is simple, sensitive, and specific, but is expensive because of the high cost of the enzyme (1). The immobilization of the enzyme onto an insoluble support permits its reuse and thereby reduces the cost of the procedure. Bais et al. (2) reported immobilization of barley oxalate oxidase onto nylon tubing and used it in a continuous-flow system for urinary oxalate determination. We describe herein immobilization of oxalate oxidase onto alkylamine glass beads and its application in discrete assays for urinary oxalate.

Zirconia-coated alkylamine glass beads (pore diameter 55 nm) were a gift from Corning Glass Works, Corning, NY, and barley oxalate oxidase (EC 1.2.3.4) was purchased from Sigma Chemical Co. (St. Louis, MO).

The glass beads were activated (3) by addition of 25 g/L glutaraldehyde (1.0 L/100 g beads) with stirring for 2 h at 30 ± 5 °C. The excess glutaraldehyde was decanted and the beads were then washed with 0.05 mol/L sodium phosphate buffer (pH 7.0). Barley oxalate oxidase was added to activated beads (1.5 g/100 g beads) and kept at 4 °C for 24 h for coupling to occur. Unbound enzyme was washed off with reaction buffer (0.01 mol/L sodium succinate buffer, pH 4.0) until no enzyme activity was detected in the washings. The enzyme retained 74% of the initial activity after immobilization, with a conjugation yield of 0.42 mg/g support. Compared with the native enzyme, the conjugated enzyme showed greater stability in the cold, had a higher optimum reaction pH, and was more resistant to inhibition by NaCl.

The reaction time at 30 °C was decreased (Table 1).

The assay of immobilized oxalate oxidase was carried out in a 25-mL conical flask wrapped with black paper. The reaction mixture, 1.9 mL of 0.1 mol/L sodium succinate buffer, pH 4.0, and 200 mg of beads with bound enzyme were preincubated at 30 °C for 2 min in a temperature-controlled water-bath-shaker. The reaction was started by adding 0.1 mL of oxalic acid (0.5 mmol/L). After 2 min of incubation at 30 °C, 1 mL of color reagent was added and kept at 30 °C for 7 min to develop the color. The reaction mixture was decanted into a cuvette. Absorbance was read in a Spectronic-20 spectrometer (Milton Roy, Rochester, NY) at a wavelength of 520 nm, and the amount of H₂O₂ generated was determined from the calibration curve for H₂O₂. The color reagent—50 mg of 4-aminophenazone, 100 mg of solid phenol, and 1 mg of horseradish peroxidase (KZ 1.0) per 100 mL of 0.4 mol/L sodium phosphate buffer, pH 7.0—was stored in an amber bottle at 4 °C. Fresh reagent was prepared every week.

Table 1. Comparative Characteristics of Soluble and Immobilized Barley Oxalate Oxidase

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Soluble enzyme (4, 7)</th>
<th>Immobilized enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>3.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Temp. for max. activity, °C</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Thermal stability at 75 °C, %</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>Eₚₙ, cal/mol</td>
<td>9140</td>
<td>11480</td>
</tr>
<tr>
<td>Time for max. activity at 30 °C, min</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Kₐₚ for oxalate, mol/L</td>
<td>4.2 × 10⁻⁴</td>
<td>3.7 × 10⁻⁴</td>
</tr>
<tr>
<td>% inhibition by NaCl (1 mmol/L)</td>
<td>57</td>
<td>18</td>
</tr>
</tbody>
</table>

Standard assay conditions were used for each determination. To study the thermal stability, we heated the enzyme at 75 °C for 40 min, cooled it, and assayed it at 30 °C.
To reuse the enzyme, the glass beads were washed with reaction buffer two or three times. One unit (1 U) of immobilized enzyme is defined as the amount of enzyme bound to glass beads that produces 1 μmol of H₂O₂ per minute under the standard assay conditions. The enzyme protein bound to the glass beads was estimated by determining the loss of protein from the solution during immobilization (4).

To determine the urinary oxalate concentration, we analyzed 24-h urine samples collected from healthy individuals; the final pH was adjusted to 2.5 by addition of HCl and they were stored at 4 °C until use. The pretreatment of urine samples was done according to Buttery and Pannall (5) with modifications: 1.0 mL of each acidified urine sample was put into a 15-mL graduated centrifuge tube, 1.0 mL of aqueous CaCl₂ (5 g/L) solution was added, and the final pH was adjusted to 6.8–7.0 by adding NaOH; 8.0 mL of ethanol was added immediately and the tubes were covered with aluminum foil and kept overnight at room temperature (25 ± 3 °C) for precipitation. The next day, tubes were centrifuged at 3000 × g for 5 min and the precipitates were dissolved in 1.0 mL of 0.1 mol/L HCl. Urinary oxalate was assayed as described for immobilized enzyme except that oxalate solution was replaced by 0.1 mL of dissolved urinary oxalate. The value of urinary oxalate was extrapolated from the calibration curve for oxalate concentration vs enzyme activity.

Color intensity varied linearly with oxalate concentration up to an absorbance of 0.11. The lower detection limit was 5 μmol/L. The mean ± SD analytical recovery of added oxalate (17.5 and 35 mg/L) was 96.7% ± 3.4%. To evaluate the accuracy of the method, we compared the oxalate results of 20 urine samples by the Sigma kit method (x) with modifications as follows: 1 mL of urine sample was diluted with an equal volume of phosphate buffer (0.1 mol/L, pH 7.5) containing EDTA (0.01 mol/L), and its pH was adjusted to 5.0–7.0. We added 200 mg of activated charcoal to the diluted urine, mixed for 5 min, and filtered it through Whatman no. 1 filter paper. To 0.05 mL of filtered urine, 0.1 mL each of oxalate reagent A (3.2 mmol of 3-(dimethylamino)benzoic acid and 0.2 mmol of 3-methyl-2-benzothiazolione in sodium succinate buffer, pH 3.5) and oxalate reagent B (3000 U of barley oxalate oxidase and 100 000 U of horseradish peroxidase per liter of distilled H₂O₂) were added and mixed. The mixture was incubated at 37 °C for 5 min and its absorbance at 590 nm was read. The regression equation for the oxalate values obtained by the Sigma kit method (x) and by the present method (y) is y = 1.13x - 0.0012 for an oxalate value of 27.0 ± 5.4 mg/L (mean ± SD).

References

Cysteine Proteinase Activity in Synovial Fluids Measured with a Centrifugal Analyzer

To the Editor:

The destruction of cartilage and bone in rheumatoid arthritis (RA) is thought to be mediated by proteolytic degradation. RA synovial fluids contain high concentrations of several proteinases, e.g., elastase (1–4), collagenase (2, 5), stromelysine (5), and lysozyme cysteine proteinases (CP) (6–8). In particular, several observations suggest potential roles for the lysozyme cathepsins B and L in cartilage degradation.

Cathepsins B and L are able to de-grade cartilage collagens type II, IX, and XI (9) and cartilage proteoglycans (10, 11). Furthermore, inhibitors of cathepsin B are able to reduce the cartilage and bone degradation in experimental induction of arthritis in rats (12, 13). Huet et al. (8) previously reported that the cysteine proteinase activity determined by using a manual fluorometric method with the substrates (N-α-benzoxycarbonyl-L-phenylalanyl-L-arginine-γ-amido-4-methylcoumarin, (Z-Phe-Arg-AMC) or Z-Arg-Arg-AMC was significantly greater in synovial fluids of RA patients than in patients with osteoarthritis (OA). Therefore, CP activities in synovial fluid may reflect a potential degradative activity against the articular cartilage of a particular joint.

We describe here an automated kinetic method for measuring the CP activity in synovial fluids by using a Cobas Fara II centrifugal analyzer (F Hoffmann-La Roche Co., Ltd., Basel, Switzerland). The CP activity was determined at 37 °C with the fluorogenic substrate Z-Phe-Arg-AMC (purchased from Bachem, Bubendorf, Switzerland), the specific inhibitor E64 [1-trans-epoxysuccinyl-leucylamido(-4-guanidino)-butane; Sigma Chemical Co., St. Louis, MO] and AMC (7-amino-4-methylcoumarin; Serva, Heidelberg, Germany) as an internal standard.

We assayed synovial fluid samples that had been drawn by aspiration from knee joints during therapy. We centrifuged the samples for 5 min at 3000 × g within an hour of collection and stored the supernates frozen at -20 °C until use. Stock and working solutions were prepared as follows: Z-Phe-Arg-AMC (10 mmol/L) was dissolved in dimethyl sulfoxide, and the working solution was obtained by diluting 50 μL of stock solution with 1950 μL of 1 g/L aqueous Brij 35 (polyoxyethylene monolauryl ether, from Serva). The stock solution of AMC (0.25 mmol/L) was diluted 100-fold with 0.3 mol/L acetate buffer, pH 5.5, just before use. The E64 stock solution (10 mmol/L) was dissolved in dimethyl sulfoxide and the working solution was obtained by diluting 5 μL of stock solution with 1515 μL of the aqueous Brij 35 to give a final concentration of 0.003 mmol/L.

The various settings of the apparatus are listed in Table 1. Briefly, the apparatus distributes successively: (a) 12.5 μL of sample and 214 μL of 0.3 mol/L acetate buffer pH 5.5, (b) 12.5 μL of the AMC working solution as internal standard, (c) 31 μL of substrate working solution, (d) 20 μL of